From the Department of Laboratory Medicine

Karolinska Institutet, Stockholm, Sweden

MOLECULAR MECHANISMS FOR TRANSCRIPTION IN MAMMALIAN MITOCHONDRIA

Martina Gaspari

Karolinska Institutet

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ABSTRACT

The circular double stranded mitochondrial genome (mtDNA), which is about 16,600 bp in humans and 16,300 bp in mice, encodes 13 of the ~90 different proteins present in the respiratory chain of mammalian mitochondria. The remaining components of the respiratory chain are encoded by nuclear genes and imported into the mitochondrial network. The genes present in mtDNA, 13 mRNAs, 22 tRNAs, and 2 rRNAs, are all essential for oxidative phosphorylation. Therefore, mtDNA replication and transcription are necessary processes for normal function of the respiratory chain and for the metabolism of the eukaryotic cell.

In this thesis, we characterized the basic mtDNA transcription machinery in mammals. We identified two novel mitochondrial transcription factors, B1 (TFB1M) and B2 (TFB2M) and for the first time we reconstituted mammalian mitochondrial transcription *in vitro* by using only pure recombinant proteins. We showed that either TFB1M or TFB2M can support promoter-specific mtDNA transcription *in vitro* if combined with mitochondrial RNA polymerase (POLRMT) and mitochondrial transcription factor A (TFAM). Studies by us and others suggest that TFB1M and TFB2M may have distinct functions: TFB2M may be primarily a transcription factor and TFB1M a methyltransferase. We identified homologues to TFB1M and TFB2M in many metazoans, including *Drosophila melanogaster*, indicating a duplication event of the TFBM gene early in evolution.

We used the recombinant *in vitro* transcription system to investigate molecular mechanisms for mtDNA promoter recognition in mammals. The transcription machineries reconstituted from mouse and human cells do not recognize the light strand promoter from the other species. By swapping transcription factors between the mouse and the human transcription machineries, we demonstrated that the observed promoter specificity is governed by POLRMT and TFAM. In contrast, TFB2M does not influence the sequence specificity in these two mammalian systems.

TFAM is a dual function protein. The protein binds without sequence specificity to the entire mtDNA molecule, thereby contributing to the formation of the nucleoid, a compact mtDNA-protein complex present in the mitochondrial matrix. TFAM is also an essential transcription factor, which interacts sequence specifically with mitochondrial promoter elements. We investigated the functional consequences of TFAM overexpression or TFAM knockdown in HeLa cells. We concluded that the mtDNA amount is directly correlated with the amount of TFAM and not with the levels

of mitochondrial transcription. Our findings indicate that TFAM has a function in mtDNA maintenance and copy number control, which is independent of its role as a transcription factor.

Finally, we used the human recombinant mitochondrial transcription system to demonstrate that the conserved sequence block II (CSB II) acts as a sequence-dependent transcription termination element *in vitro*. In mitochondria, transcription generates RNA primers needed for initiation of leading (heavy) DNA strand synthesis. We found that transcription from the light strand promoter is prematurely terminated at CSB II, downstream of the promoter. This premature termination event is critically dependent on the exact CSB II sequence. The 3'-ends of the prematurely terminated transcripts coincide with the major points of RNA to DNA transition in leading strand DNA replication. These findings suggest that primer formation is directed by specific mtDNA sequence elements and thereby provide a novel model for initiation of mtDNA replication in mammalian cells.

Keywords: mitochondria, oxidative phosphorylation, mtDNA, transcription, TFAM, TFB1M, TFB2M, POLRMT, promoter recognition, CSB II.

LIST OF PUBLICATIONS

The present thesis is based on the following papers. These papers will be referred by their roman numerals.

I. Falkenberg M, **Gaspari M**, Rantanen A, Trifunovic A, Larsson NG, Gustafsson CM.
Mitochondrial transcription factor B1 and B2 activate transcription of human mtDNA.
Nat Genet. 2002 Jul; 31(3): 289-94

II. Rantanen A, **Gaspari M**, Falkenberg M, Gustafsson CM, Larsson NG.
Characterization of the mouse genes for mitochondrial transcription factors B1 and B2.
Mamm Genome. 2003 Jan; 14(1): 1-6

III. **Gaspari M**, Falkenberg M, Larsson NG, Gustafsson CM.
The mitochondrial RNA polymerase contributes critically to promoter specificity in mammalian cells.
EMBO J. 2004 Nov 24:23(23): 4606-14

IV. Kanki T, Ohgaki K, **Gaspari M**, Gustafsson CM, Fukuoh A, Sasaki N, Hamasaki N, Kang D.
Architectural role of mitochondrial transcription factor A in maintenance of human mitochondrial DNA.
Mol Cell Biol. 2004 Nov; 24(22): 9823-34

V. Pham XH, Farge G, Shi Y, **Gaspari M**, Gustafsson CM, Falkenberg M.
Conserved sequence box II directs transcription termination and primer formation in mitochondria.
J Biol Chem. 2006 Aug 25; 281(34): 24647-52

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LIST OF ABBREVIATIONS

Abf2	ARS-binding factor2
ANT2	Adenine nucleotide translocator 2
ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
bp	Base pair
cDNA	Complementary DNA
CSB	Conserved sequence block
D-loop	Displacement loop
DNA pol γ	DNA polymerase γ
DPE	Distal promoter element
E	Embryonic day
GFP	Green fluorescent protein
GTP	Guanosine triphosphate
H	Heavy strand
HeLa cells	Human epithelial cells from cervical cancer
HMG	High mobility group
HSP	Heavy strand promoter
IT_L	Initiation of light strand transcription
kb	Kilobase
Da, kDa	Dalton, KiloDalton
L	Light strand
LSP	Light strand promoter
mRNA	Messenger RNA
MELAS	Mitochondrial myophathy, encephalopathy, lactic acidosis and stroke-like episodes syndrome
mtDNA	Mitochondrial DNA
mtSSB	Mitochondrial single-stranded binding protein
NRF-1/NRF-2	Nuclear respiratory factor-1/-2
OXPHOS	Oxidative phosphorylation
O_H	Origin of heavy strand replication
O_L	Origin of light strand replication
PCR	Polymerase chain reaction
POLRMT	Mitochondrial RNA polymerase
PPE	Proximal promoter element
PT	Prematurely terminated transcript
RO	Run off transcript
rRNA	Ribosomal RNA
RNase MRP	RNase mitochondrial RNA processing
ssDNA	Single-stranded DNA
tRNA	Transfer RNA
TAS	Termination associated sequences
TFAM	Mitochondrial transcription factor A
TFB1/2M	Mitochondrial transcription factor B1 or B2

INTRODUCTION

In 1962 Rolf Luft reported for the first time a role for mitochondria in human pathologies (Luft et al., 1962). It was a big breakthrough, since no one had previously shown that mitochondrial malfunction could lead to human diseases. Today, the existence of different mitochondrial disorders is well established (Wallace, 1992). Patients present a variety of clinical problems often involving heart, skeletal muscle and the central nervous system. Mitochondrial diseases are inherited either in a maternal or a Mendelian way (Wallace, 1999). This inheritance complexity arises from the fact that mitochondria are semi-autonomous organelles. Most mitochondrial proteins are in fact nucleus encoded. Thus, functional mitochondria require polypeptides from both the nuclear and the mitochondrial genomes. Mutations in either genome may therefore cause mitochondrial dysfunction (Larsson and Clayton, 1995). Pathogenic mitochondrial DNA mutations were first reported in 1988 (Holt et al., 1988; Wallace et al., 1988); one year later Zeviani M. *et al*. published evidence that nuclear genes also could be involved in mitochondrial disorders (Zeviani et al., 1989). Over the years, the number of mitochondrial DNA mutations linked to human diseases has continuously increased (Larsson and Clayton, 1995) and continues to rise. Furthermore, mitochondrial dysfunction may play a role in common forms of neurodegenerative diseases, such as Parkinson's and Alzheimer's and even in the normally occurring aging process (Trifunovic et al., 2004). All this has created a major interest among scientists to learn more about the origin and functions of these fascinating organelles.

THE MITOCHONDRIAL ORIGIN

Studying the origin of mitochondria is a challenging task. In fact, we are immediately confronted with an extraordinary variation in size, structure and mode of expression of mitochondrial DNA (mtDNA) within the four traditional eukaryotic kingdoms (Animalia, Plantae, Fungi and Protista) (Wallace, 1982). Vertebrate mtDNA is an "extreme example of genetic economy" (Attardi, 1985), consisting of structural genes which are joined one to another, with few or no spacer nucleotides between them. One major regulatory region, the displacement loop (D-loop) is present in the genome and it shows size variation in different species. Introns, which are missing in animal mtDNA,

are found in fungi and plants. In particular, plant mitochondrial genomes are the largest and most complex mtDNAs known (Newton, 1988). Genes are scattered and the genome size can reach up to 2400 kbp in *Cucumis melo.* The genome, denoted the master chromosome, can even be resolved into a number of sub genomic molecules created by intramolecular recombination (Lonsdale et al., 1984). The presence of these predicted circular forms were shown in 1988 (Palmer, 1988). Chloroplast sequences are widely distributed in plant mtDNA, mostly in random fashion. This suggested a transfer of genetic information from chloroplasts to mitochondria in the course of evolution, with some of those sequences (tRNAs in particular) still in use. Thus, the plant mitochondrial genome may be considered an evolutionary mosaic, with evidence for the acquisition of genetic information from different sources over time (Nugent and Palmer, 1988). The diversity presented above has made it very difficult for evolutionists to discern the pathways of mitochondrial genome evolution. Today, the generally accepted explanation for the origin of the mitochondrial genome is the endosymbiont hypothesis (Gray, 1989; Gray and Doolittle, 1982). This hypothesis proposes that mitochondria originated in evolution as a single invasion of an Archea-type host by a α-proteobacterium over 2 billion years ago. Most likely this α-proteobacterium was related to what we today know as *Rickettsia prowazekii*. This is an obligate intracellular parasite, which causes epidemic typhus in humans. Its genome contains 834 protein-coding genes, which represents 75.4% of the 1.1Mbp DNA sequence. Some of these genes encode components for aerobic respiration and ATP production (Andersson et al., 1998). It seems that most of the genes found in mtDNA today represent genetic information retained from the original endosymbiont. Thus, nuclear and mitochondrial genomes derive from two distinctly separate lineages. They were independent for some time until, probably driven by metabolic requirements, the genomes became united in a single cell. From that time point, the mitochondrial proteome displayed both reductive and expansive processes to acquire the contemporary structure (Andersson et al., 2002). We cannot exclude that some genetic information was acquired later in time after the symbiosis had been established. A witness of this type of event is the brown alga *Pylaiella littoralis*. This alga contains an entire T7-phage-type RNA polymerase gene integrated in a mitochondrial genome (Rousvoal et al., 1998). Together with this gene, it also contains σ-70 proteobacterial promoter regions (Delaroque et al., 1996), which indicate traces of the ancestral $\alpha_2\beta\beta'\sigma$-70 proteobacterial RNA polymerase (Ding and Winkler, 1990). It remains an open question when the phage-encoded RNA polymerase was acquired. It could have

either pre-existed in the ancestral α-proteobacterium or have been recruited early in the evolution of mitochondria. In any case, the mitochondrial genome of *P.littoralis* suggests that both the proteobactrial and the phage-type RNA polymerases have coexisted sometimes during evolution. In *P. littoralis* the phage-type RNA polymerase is still encoded in mitochondria whereas in animal, fungi and plants it has been transferred to the nucleus.

MITOCHONDRIA ARE DYNAMIC ORGANELLES

In 1890 Altmann described a technique to stain mitochondria and postulated their metabolic and genetic autonomy. He was impressed by their morphological resemblance with bacteria and called them "elementary organisms" to indicate his belief that they were elementary units existing in all cells (Cowdry, 1953). Over the years we have learnt that the morphology of these organelles is different in different cell types and organisms: small, bean-shaped, elongated tubules or highly branched reticula. Mitochondria are not considered to be distinct particles anymore but rather a dynamic network. They can change both shape (elongation, shortening, branching, buckling, swelling) and location inside living cells (Bereiter-Hahn and Voth, 1994). Alterations in mitochondrial shape at a given time are controlled by two main processes: mitochondrial fission and fusion (Chen and Chan, 2005). These two processes occur during normal cell growth, cellular differentiation and development (Yaffe, 1999). For example, during spermatogenesis in insects, fusion generates giant mitochondria that associate with the axoneme of the sperm flagella (Dallai et al., 2005; Hales and Fuller, 1997). The generation of such large, interconnected mitochondrial compartments is thought to facilitate the distribution of energy and metabolites as well as chemicals and electrical signals throughout these cells (Ichas et al., 1997). The protein machineries that drive fission and fusion can remodel the double membrane structure of mitochondria: the outer membrane is separated from the inner membrane by the intermembrane space and the inner membrane is folded to form cristae. Fission and fusion must thus accomplish synchronised remodelling of both membranes to separate or fuse organelles (Osteryoung and Nunnari, 2003). Recently, it was discovered that the optic atrophy type 1 gene (OPA1), encoding a protein involved in organelle fusion, is mutated in patients with autosomal dominant optic atrophy (Alexander et al., 2000; Delettre et al., 2000). Optic atrophy type 1 is an optic

neuropathy occurring in 1 in 50,000 individuals leading to progressive loss of retinal ganglion cells and blindness. OPA1 encodes a dynamin-related GTPase localized to mitochondria, homologous to the *Saccharomyces cerevisiae* Mgm1p protein (Ishihara et al., 2006; Olichon et al., 2002). Mgm1p is localized to the intermembrane space and is required together with Fzo1p and Ugo1p (outer membrane proteins) for mitochondrial fusion in yeast. Cells lacking any of these GTPases contain many small mitochondrial fragments rather than the branched mitochondrial tubules seen in wild-type cells. The fragmentation of mitochondria in *fzo1, ugo1 and mgm1* mutants is a consequence of ongoing mitochondrial division in the absence of fusion (Sesaki et al., 2003; Wong et al., 2000). Recent studies have shown that Ugo1p, Fzo1p and Mgm1p either coassemble or interact with each other in pairs (Sesaki and Jensen, 2004). The human homologs for Fzo1p are mitofusins Mfn1 and Mfn2 (Santel, 2006; Santel et al., 2003; Santel and Fuller, 2001). In yeast, the large dynamin-related GTPase Dnm1p (Drp1/Dlp1 in humans) promotes fission, together with Fs1p, Mdv1p and Caf4p (Schauss et al., 2006). These proteins are localized on the cytoplasmic face of the outer mitochondrial membrane and work together to generate local constriction in the mitochondrial tubule. Two recent studies showed that mammalian Drp1 assembles to form rings *in vitro*, suggesting that a similar process occurs *in vivo* (Smirnova et al., 2001; Yoon et al., 2001). GTP hydrolysis might induce a conformational change of these assembled Dnm1p ring-structures, resulting in tubule scission at the constricted sites. Unlike Mdv1p and Caf4p, Fis1p homologues have been identified in higher eukaryotes, including humans (James et al., 2003).

THE MITOCHONDRIAL GENOME

Structure of the human mitochondrial genome

The mitochondrial DNA genome (mtDNA) is contained in the matrix, which is the space enclosed by the inner mitochondrial membrane. Together with mtDNA, the matrix contains mitochondrial ribosomes, tRNAs and a highly concentrated mixture of hundreds of enzymes, required for mitochondrial function. Mitochondria are polyploid, with hundreds to thousands of copies of its genome in each cell. It was early recognized that mtDNA does not distribute homogenously within the mitochondrial compartment, but concentrates in structures called nucleoids, most often associated

with the mitochondrial inner membrane (Dimmer et al., 2005; Echeverria et al., 1991; Hall et al., 1975; Kuroiwa, 1982). The functional basis for these nucleoid structures and their association with the membrane is not yet clear, but they may have roles in inheritance, segregation, replication, transcription or perhaps recombination. The most significant progress in understanding nucleoids has been achieved in the yeast *Saccharomyces cerevisiae*. In *S. cerevisiae*, each mitochondrial nucleoid contains several genomes and each cell contains from 5 to 20 nucleoids depending on growth conditions and genetic background (Lockshon et al., 1995; MacAlpine et al., 2000). Formaldehyde cross-linking experiments have revealed at least 20 protein components of the yeast mitochondrial nucleoid, including Abf2 (the abundant high-mobility group DNA binding protein), aconitase (Chen et al., 2005), Ilv5, Mm1p (Hobbs et al., 2001), Mdm10p, Mdm12p (Boldogh et al., 2003). Some of these proteins seem to mediate contacts between nucleoids and cytoskeletal components important for mtDNA inheritance (Kaufman et al., 2000). Ideally, the conditions chosen for cross-linking, solubilization and purification should allow the retention of "true" components and lead to loss of "unspecific" contaminants. However, in real life it is difficult to establish such "ideal" conditions and the distinction between components and contaminants requires localization and functional characterization of newly identified molecules (Malka et al., 2006). Recently, Bogenhagen and co-workers reported the purification of mtDNA nucleoids from cultured human HeLa cells and were able to identify the associated proteins. They obtained two subsets of complexes, which differ in their sedimentation velocity. The more rapidly sedimenting form was closely associated with cytoskeletal proteins, reminiscent of the yeast nucleoids summarized above. The more slowly sedimenting form lacked extensive interaction with cytoskeletal elements. They used this fraction as starting point for immunoaffinity purification using antibodies directed against two abundant mtDNA binding proteins, mitochondrial transcription factor A (TFAM) or mitochondrial single-stranded binding protein (mtSSB). With both antibodies, a set of around 20 similar polypeptides was detected including proteins known to be involved in mtDNA maintenance (Bogenhagen et al., 2003; Wang and Bogenhagen, 2006).

Gene content and organization

Human mitochondrial DNA is a double-stranded closed circular molecule of about 16.6 kilo base (kb) length. In density gradients the double-stranded molecule can be

separated into a light strand (L) and a heavy strand (H). The observed difference is due to uneven nucleotide content of the two strands: the H-strand is guanine rich, whereas the L-strand is guanine poor. There are no introns in the genome and the 37 genes are highly compacted; they encode 13 mRNAs (all necessary subunits of the respiratory chain enzyme complexes), 22tRNAs and 2rRNAs (16S rRNA and 12S rRNA) required for translation (Anderson et al., 1981). All of the other mitochondrial protein components are encoded in the nucleus, synthesised in the cytoplasm and imported into mitochondria by using special targeting sequences, some of which are cleaved after import (Shadel and Clayton, 1997). Most of the genetic information of mtDNA is encoded on the heavy strand, including genes for both rRNAs, 14 tRNAs and 12 polypeptides; the light strand encodes 8 tRNAs and a single polypeptide, ND6. Some of the protein genes overlap and many termination codons are generated post-transcriptionally by polyadenylation of the processed mRNAs (Montoya et al., 1981; Temperley et al., 2003). The tRNAs genes are scattered among the other genes and play an important role in RNA processing. They act as signals for the processing enzymes after acquiring the cloverleaf structure on the nascent RNA chains. There are only two longer non-coding regions in mammalian mtDNA and they contain most of the known regulatory functions. The main non-coding region is called the displacement loop (D-loop) and is situated between the genes for $tRNA^{Phe}$ and $tRNA^{Pro}$. This region is about 1kb and gets its name from the special three-stranded structure, in which a short 500-700 nucleotides strand (called 7S DNA), complementary to the L-strand, displaces the H strand. This D-loop region has evolved as the major control site for mtDNA expression, containing the leading-strand origin of replication (O_H) and the major promoters for transcription of both strands (Taanman, 1999). Two overlapping units transcribe the H-strand *in vivo* (Montoya et al., 1983). Heavy strand $promoter_1$ (HSP_1) is located 19 nucleotides (nt) upstream of the $tRNA^{Phe}$ and is responsible for the synthesis of the two ribosomal RNAs, $tRNA^{Phe}$ and $tRNA^{Val}$. This transcription unit operates much more frequently than heavy strand $promoter_2$ (HSP_2) and its activity is linked to a transcription termination event taking place immediately downstream 16S rRNA, inside the gene for $tRNA^{Leu}$ (Asin-Cayuela et al., 2005; Daga et al., 1993; Kruse et al., 1989; Martin et al., 2005). The second heavy strand promoter (HSP_2) is located close to the 12S rRNA 5'-end and initiates transcription of a polycistronic RNA molecule covering most of the H-strand. Thus, differential regulation of rRNA versus mRNA is obtained through the initiation of H-strand transcription at these two alternative sites. The light strand promoter (LSP) is situated about 150 bp away from

HSP_1 and initiation of transcription at nucleotide 407 (IT_L) creates a single long polycistronic RNA containing eight tRNAs and the ND6 mRNA (**Figure 1**) (Montoya et al., 1982).

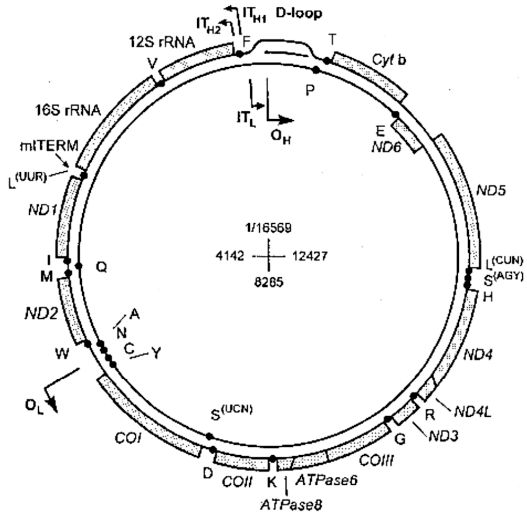

Figure 1. Map of the human mitochondrial genome

The outer circle represents the H-strand, containing the majority of the genes; the inner circle represents the L-strand. The D-loop is shown as a three-stranded structure. The origins of H-strand (O_H) and L-strand (O_L) replication and the direction of DNA synthesis are indicated by long bent arrows; the initiation of transcription sites (IT_L, IT_{H1}, IT_{H2}) and the direction of RNA synthesis are denoted by short bent arrows. The binding site for the mitochondrial transcription terminator (mtTERM) is indicated. The 22 tRNAs are depicted by dots, the genes coding for the two rRNA species (12S and 16S) and the 13 protein coding genes are depicted by shaded boxes (Taanman, 1999).

Mechanisms for controlling H_2 and L-strand termination must exist, but the precise location of termination sites and mechanisms for termination are at the moment unknown. It should also be noted that *in vitro* transcription studies have failed to show any detectable H_2 initiation events. This has lead to the proposal that there may be only one major transcription initiation point for each mtDNA strand (HSP and LSP). According to this model, transcription from H_1 would generate both mRNAs and rRNAs, and regulation of the rRNA/mRNA transcript ratios would be controlled at the termination site (Clayton, 1992). A regulatory element is positioned upstream of the transcription initiation site of both promoters, at position –12 to –39 bp. This regulatory element is binding a transcription factor, called mitochondrial transcription factor A

(mtTFA or TFAM), which is essential for mitochondrial transcription initiation and maintenance of mtDNA (Fisher and Clayton, 1988; Fisher et al., 1987; Larsson et al., 1998). Mapping of RNA and DNA species in the D-loop region of human and mouse mtDNA have suggested that short mitochondrial transcripts, originated at LSP, serve as primers for the initiation of synthesis of nascent H-strands. Thus, replication of mammalian mtDNA appears to be intimately linked with mitochondrial transcription (Lee and Clayton, 1998; Xu and Clayton, 1996). Sequence comparisons in vertebrates have revealed three conserved sequence blocks (termed CSB I, CSB II, CSB III) downstream of LSP. CSB II increases the stability of an RNA-DNA hybrid and transitions from the RNA primer to the newly synthesized DNA have been mapped to sequences within or near CSB II. Because of their location it has been speculated that CSB I, II and III direct the precise cleavage of primary transcripts to provide the appropriate primer species (Chang and Clayton, 1985; Xu and Clayton, 1995). The D-loop also contains short (15bp) sequences conserved in vertebrates and called TAS (termination associated sequences). These sequences seem to be associated with the 3'-ends of arrested D-loop strands (**Figure 2**) (Doda et al., 1981; Fernandez-Silva et al., 2003; Madsen et al., 1993). Finally, the second non-coding region is the origin of light strand replication (O_L), which is located far away from the H-strand origin and nested in a cluster of five tRNAs genes (Hixson et al., 1986; Wong and Clayton, 1985).

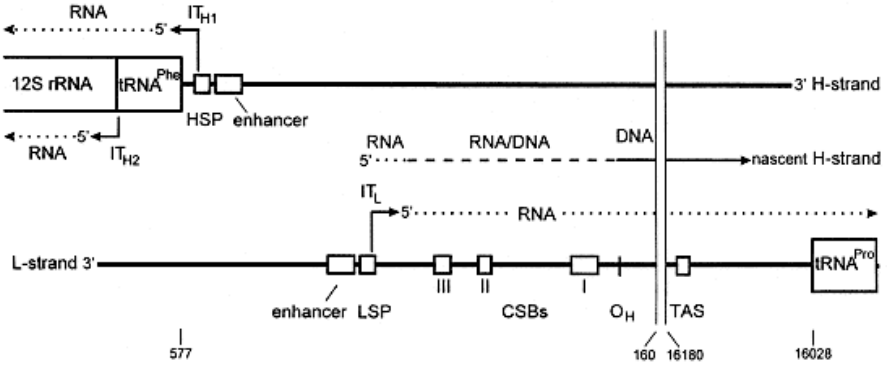

Figure 2. The D-loop region controls replication and transcription of mtDNA

In the D-loop region two major transcription initiation sites are present. Transcription initiation site IT_{H1}, encompassed by the H-strand promoter (HSP), directs the

transcription of the H-strand, whereas transcription initiation site IT_L, encompassed by the L-strand promoter (LSP), directs the transcription of the L-strand. A second minor transcription initiation site (IT_{H2}) for H-strand transcription is located in the gene for $tRNA^{Phe}$ near the boundary with the 12S rRNA gene. Enhancer elements upstream of the HSP and LSP that are known to bind the mitochondrial TFAM are indicated. A short RNA transcript originating at IT_L serves as a primer for replication of the (leading) H-strand. Transition from RNA to DNA occurs within the dashed line, in the region around the conserved sequence blocks (CSBs) I, II and III. O_H is the origin of H-strand synthesis. Short DNA strands that are part of the triplex D-loop structure terminate near the termination-associated sequence (TAS) (Taanman, 1999).

MITOCHONDRIAL ENERGY PRODUCTION

Adenosine 5'-triphosphate (ATP), discovered in 1929 by Karl Lohmann, is the most important "free-energy currency" molecule in living organisms. In fact, dephosphorylation of ATP to ADP and inorganic phosphate (P_i) is a very effective way for the body to cause vital non-spontaneous reactions to occur. As these non spontaneous reactions occur, ATP is used up. In a typical cell, an ATP molecule is used within a minute of its formation. During strenuous exercise, the rate of utilization of ATP is higher. Hence, the supply of ATP must be regenerated. One of the principal energy-yielding nutrients in our diet is glucose, a simple six-carbon sugar. The complete breakdown of glucose to CO_2 occurs in two processes: glycolysis and the citric-acid cycle. Glycolysis and the citric-acid cycle produce a net total of only four ATP or GTP molecules per glucose molecule. This yield is far below the physiological requirements of the eukaryotic cell. To achieve higher ATP generation (**Figure 3**) the cell uses a process called oxidative phosphorylation (OXPHOS), which is carried out by the mitochondrial respiratory chain, a series of five enzyme complexes embedded in the inner mitochondrial membrane (Hatefi, 1985; Saraste, 1999).

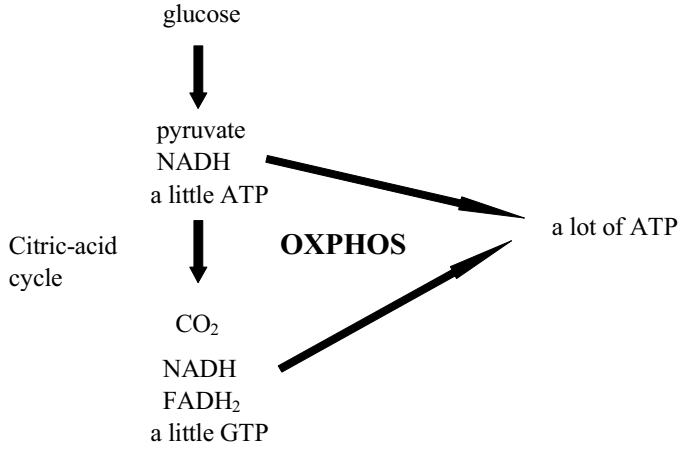

Figure 3.

This flow-chart shows the major steps involved in breaking down glucose from the diet and converting its chemical energy in the phosphate bonds of ATP.

Glycolysis and the citric-acid cycle generate other products besides ATP and GTP, namely NADH and $FADH_2$. During oxidative phosphorylation electrons are transferred from NADH or $FADH_2$ to molecular oxygen via the five enzyme complexes. Protons are pumped from the mitochondrial matrix into the intermembrane space as a result of this flow of electrons. This generates a proton gradient and a transmembrane electrical potential across the membrane. This is a form of potential energy, denoted proton-motive force (Mitchell, 1961; Racker, 1980). The protons flow back into the mitochondrial matrix via a large protein complex, called ATP synthase. One glucose molecule produces 32 ATP: 2 ATP are generated in the glycolitic pathway, the remaining 30 through OXPHOS. The five enzyme complexes (**Table 1**) of the respiratory chain consist of ~ 90 subunits, 13 of which are encoded in the mitochondrial genome. Complex II is the only one that does not contain any mitochondrial polypeptides and thus is dependent only on correct nuclear gene expression. Complexes II-V have been crystallized to a certain extent and important mechanistic conclusions have been drawn.

Table 1. Enzyme information

		kDa
Complex I	NADH dehydrogenase	800
Complex II	Succinate dehydrogenase	140
Complex III	Cytochrome C oxidoreductase	250
Complex IV	Cytochrome C oxidase	170
Complex V	ATP synthase	380

Complex I is the largest of all complexes, has a L-shaped structure and seven subunits are encoded by mtDNA (Grigorieff, 1998; Guenebaut et al., 1998). Electrons are fed by NADH into Complex I or by succinate into Complex II and are then passed to Coenzyme Q (called also ubiquinone) (Yankovskaya et al., 2003). Ubiquinone is a small, lipid soluble molecule that carries electrons to Complex III.

In Complex III, electrons are transferred to cytochrome c, a peripheral membrane protein bound to the outer face of the inner membrane, which then carries electrons to Complex IV, where they are finally transferred to O_2. The free energy derived from the passage of electrons through complexes I, III, and IV is harvested by being coupled to the synthesis of ATP. ATP is produced by Complex V, which is organized into two structurally distinct components, F_0 and F_1. The F_0 portion spans the inner membrane and provides a channel through which protons are able to flow back from the intermembrane space to the matrix. The energetically favorable return of protons to the matrix is coupled to ATP synthesis by the F_1 subunit, which catalyzes the synthesis of ATP from ADP and phosphate ions. In particular, the flow of protons through F_0 drives the rotation of a part of F_1, which acts as a rotary motor to drive ATP synthesis (Elston et al., 1998; Yoshida et al., 2001) (**Figure 4**).

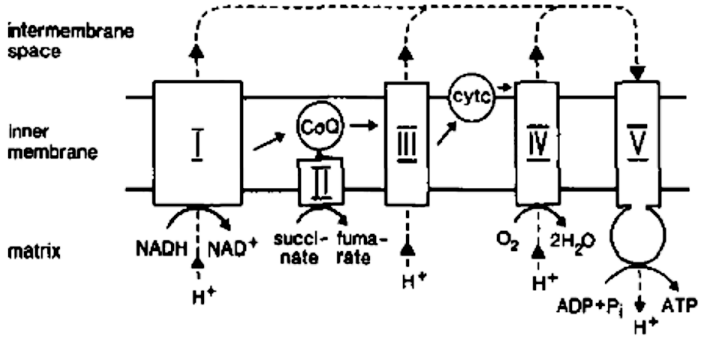

Figure 4. Schematic illustration of the respiratory chain.

The respiratory chain consists of 5 complexes, designated Complex I-V. Complex II does not translocate protons and is completely nucleus-encoded (Larsson and Clayton, 1995).

Lately, different observations suggest that these respiratory complexes are organized in supercomplexes. These supermolecular structures, also called "respirasomes" were observed in mitochondria of *S. cerevisiae* (Schagger and Pfeiffer, 2000), plants (Eubel et al., 2004; Eubel et al., 2003; Krause et al., 2004) and mammals (Schafer et al., 2006; Schagger and Pfeiffer, 2001).

MITOCHONDRIAL BIOGENESIS

Mitochondrial biogenesis requires gene products from two physically separated genomes: one contained within the organelle and the other within the nucleus. This implies that a coordinate regulation of these two genetic systems is necessary for mitochondrial function in the cell (Garesse and Vallejo, 2001). Recognition sites for nuclear transcription factors have been found in a number of nuclear genes whose products contribute to mitochondrial respiration. Among these factors, nuclear respiratory factor 1 (NRF-1) and nuclear respiratory factor 2 (NRF-2) have been shown to have positive effects on several respiratory gene promoters (Gleyzer et al., 2005; Virbasius and Scarpulla, 1994). NRF-1 was first identified as a transcriptional activator of the rat cytochrome c gene (Evans and Scarpulla, 1989); targeted disruption of the *Nrf1* gene in mice causes a dramatic decrease in the amount of mtDNA, and a preimplantation lethal phenotype (Huo and Scarpulla, 2001). NRF-1, together with NRF-2, binds to specific sites of TFAM gene promoter, being the major determinants of TFAM gene transcription. Together with NRF-1 and NRF-2, a third factor, SP1 plays a central role in the transcriptional expression of several functionally diverse OXPHOS promoters, such as adenine nucleotide translocator 2 (ANT2), cytochrome c, TFAM, and F_1-ATPase β-subunit (Li et al., 1996b). All these four promoters contain multiple, proximal SP1-activating elements that account for 50% or more of the transcription activation by SP1. Distal Sp1 elements are less defined and are more promoter-specific. SP1 acts on ANT2 and F_1-ATPase β-subunit promoters as an activator, but also as a repressor. This role of Sp1 in both the positive and negative regulation of OXPHOS promoters is unique (Li et al., 1996a; Zaid et al., 1999). Another family of key regulators controlling mitochondrial respiration and mass are the peroxisome proliferators-activated receptor γ (PPARγ) coactivators (PGCs): PGC1α, PGC1β and PGC1α-related coactivator (PRC). PGC1α was originally identified as an interacting partner of the nuclear hormone receptor PPARγ by two-hybrid screening (Puigserver et al., 1998). Forced expression of PGC1α was shown to induce mitochondrial biogenesis in adipocyte and myogenic cell lines and to coactivate the transcription function of NRF-1 on the TFAM promoter (Wu et al., 1999). PGC1α was also found to interact with PPARα in the transcription of nuclear genes encoding mitochondrial fatty acid oxidation enzymes (Vega et al., 2000). PGC1α is predominantly expressed in tissues with abundant mitochondria such as heart, brown

adipose tissue, skeletal muscle, kidney, liver and it is regulated by certain physiological conditions. PRC has recently been described and unlike PGC1α it is ubiquitously expressed. PRC and PGC1α are indistinguishable in their interaction with NRF-1 and in the coactivation of NRF-1 genes. Thus, it is likely that they provide complementary functions in governing mitochondrial biogenesis. PGC1α clearly mediate thermogenic responses, while PRC is most responsive to proliferative signals and is regulated according to the cell cycle (Andersson and Scarpulla, 2001). PGC1β displays greater homology to PGC1 than to PRC. In fact it is highly expressed in heart and brown adipose tissue and is induced by fasting (Lin et al., 2002).

MITOCHONDRIAL DNA REPLICATION

Mitochondrial DNA replication occurs independently of the cell cycle and is not correlated with nuclear DNA replication (Bogenhagen and Clayton, 1977). Recent work has shown that it is possible to reconstitute a minimal mtDNA replisome *in vitro* with recombinant proteins (Korhonen et al., 2004). The mitochondrial DNA polymerase (POLγ) is a heterotrimeric complex consisting of one catalytic (POLGA) subunit and two processivity (POLGB) subunits. POLγ together with TWINKLE DNA helicase (Korhonen et al., 2003) forms a processive replication machinery that synthesizes single-stranded DNA (ssDNA) molecules of about 2 kb in length. If mitochondrial ssDNA binding protein (mtSSB) is added, DNA products of about 16kb are generated, which represent the size of the mammalian mtDNA molecule. These data provide a new biochemical basis for investigating the way mtDNA replicates, an intensely discussed topic over the years. The conventional model for mtDNA replication is called the strand-asymmetric model (or Clayton model) (Clayton, 1982); in year 2000, the symmetric model (or Holt model) was proposed on the basis of neutral/neutral two dimensional (2-D) gel electrophoresis experiments (Holt et al., 2000). The two models have not yet been reconciled and are at present not compatible with each other.

The strand-asymmetric model (Clayton model)

In this model two origins of replication exist, one for each mtDNA strand: the origin of heavy-strand synthesis (O_H) and the origin of light strand synthesis (O_L). DNA

synthesis begins at the O_H, utilizing a RNA primer transcribed from the light-strand promoter (LSP). H-strand DNA elongation continues around the entire length of the genome, with parental H strand displacement. Interestingly, only a few initiation events at O_H actually result in the synthesis of a full-length genome; nearly 95% of initiation events are terminated at the TAS sequences. Hence, the site where H-strand DNA synthesis is either prematurely terminated or allowed to continue, functions as regulatory domain for mtDNA replication (Madsen et al., 1993). When two-thirds of the parental H strand has been displaced, the origin of light-strand replication (O_L) becomes exposed as a single-stranded unit. Synthesis of L-strand DNA begins and proceeds in a direction opposite to that of H-strand replication (Wong and Clayton, 1985). How the two circular duplex DNAs segregate at the termination of daughter-strand synthesis remains unclear. Anyhow, the two daughter molecules can be identified as: the α daughter, in which duplex synthesis is complete, the β daughter, in which synthesis of the daughter L strand must proceed from O_H to O_L before the replication is complete. This ~5.4kb gap in the β daughter is rapidly filled and both daughter molecules are processed to a closed circular form at approximately the same rate (Berk and Clayton, 1974; Clayton, 1982). Superhelical turns are introduced into the two daughter molecules and the final product of this event is called C mtDNA. The half-life of C mtDNA is less than an hour and a new D loop is immediately generated. The formation of D loop is an aggressive process and most mtDNAs are of D-loop type (Bogenhagen and Clayton, 1978) (**Figure 5**).

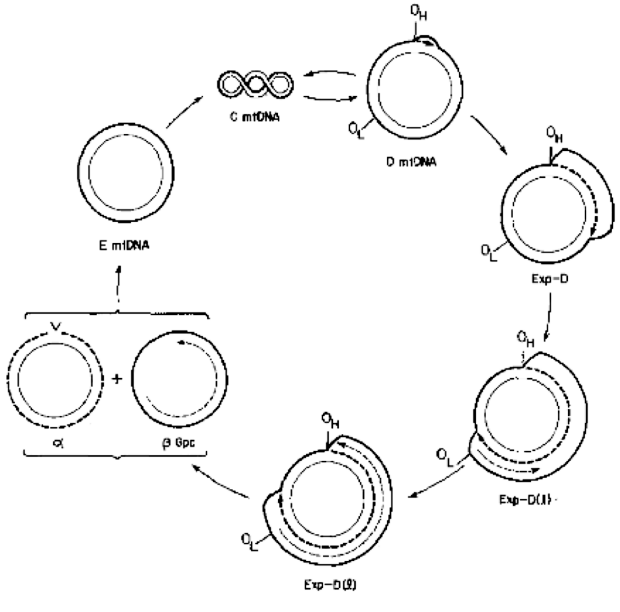

Figure 5. **The strand-asymmetric model of replication (Clayton model)**

Thick solid lines: parental heavy (H) strands. Thin solid lines: parental light (L) strands. Thick dashed lines: daughter H strands. Thin dashed lines: daughter L strands. The order of replication is clockwise starting at D mtDNA. O_H and O_L: origins of H- and L-strand synthesis. The double arrows reflect the metabolic instability of D-loop strands and consequent equilibrium between D mtDNA and C mtDNA. Expanded D-loop replicative intermediates are termed Exp-D prior to initiation of L-strand synthesis and Exp-D (ℓ) after initiation of L-strand synthesis. β Gpc: gapped circular β daughter molecule (Clayton, 1982).

The strand-symmetric model (Holt model)

The strand symmetric model was recently proposed; it was described and corrected in several steps (Bowmaker et al., 2003; Holt et al., 2000; Yang et al., 2002). Using 2-D gel electrophoresis, a variety of double-stranded replication intermediates (RI) were defined and they all pointed towards a conventional coupled leading and lagging strand replication. Some of these double-stranded RIs contained large regions of ribonucleotides, in agreement with multiple priming sites during lagging-strand synthesis. Replication of mtDNA starts at multiple sites (*Ori*) inside the initiation zone and subsequently proceedes bidirectionally. O_H provides fork arrest and replication is then restricted to one direction only. This model is exclusively based on 2D-gel methods and other independent experimental approaches are necessary to further support it.

MITOCHONDRIAL DNA TRANSCRIPTION

Transcription of mitochondrial DNA depends on a RNA polymerase distinct from the nuclear RNA polymerases. It is encoded as a single-subunit enzyme in the nucleus and then imported in the organelle. It is homologous to the RNA polymerases of bacteriophages T7 and T3 RNA (Masters et al., 1987), which are highly processive enzymes with a molecular weight of ~ 98 kDa. T7 and T3 polymerase can alone perform all of the functions required for transcription: promoter recognition, initiation, elongation and termination. They are 82% identical at the amino acid level (Davanloo et al., 1984; McGraw et al., 1985); despite their similarities, they are highly specific and neither of them can initiate transcription from the heterologous promoter (Sousa et al., 1993). Mitochondrial RNA polymerases have now been purified from three organisms:

humans, *Xenopus laevis* and *Saccharomyces cerevisiae*. Their characterization has helped to discover other protein components required for transcription in mitochondria. It appears that these protein components together with promoter elements have diverged considerably during evolution. However, one homologous protein, mitochondrial RNA polymerase, is involved in transcription initiation in all systems.

Transcription initiation in yeast

Unlike the two closely linked promoters in human mtDNA, multiple promoters are found in yeast, which are distributed throughout the genome. There are at least thirteen sites of transcription initiation and they were all mapped *in vitro* by using guanylyltransferase (Christianson and Rabinowitz, 1983). 5' RNA ends generated by de novo synthesis can be distinguished from 5' RNA ends generated by RNA processing, as de novo formed RNAs are capped at their 5' end and thus react with guanylyltransferase. At each of these thirteen sites, a well-conserved sequence motif of 11 nt (ATATAAGTAPuTA) is present and the initiation of transcription occurs at the underlined A residue. The importance of this conserved sequence motif has been confirmed by deletion and point mutation analyses in *in vitro* transcription systems (Biswas et al., 1985; Biswas et al., 1987) (**Figure 6**).

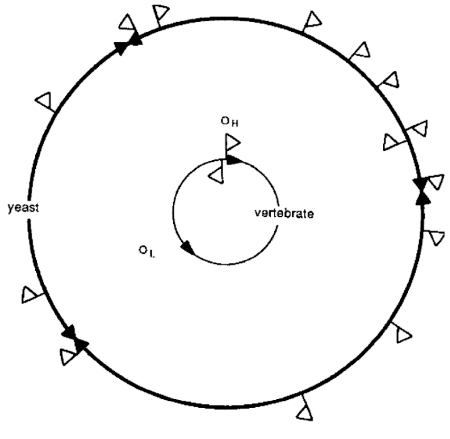

Figure 6. Comparison of replication and transcription control elements between vertebrate and yeast mtDNA.

Major promoters in both genomes are represented by open flags and replication origins for each of the two DNA strands with black triangles. Flags and triangles point in the direction of transcription and replication. O_H and O_L designate the origins of replication

for the heavy and light DNA strands (Schinkel and Tabak, 1989). Mitochondrial genes in yeast are transcribed individually or as part of a multicistronic transcript encompassing many genes. Competition experiments *in vitro* and RNA pulse labelling *in vivo* show that rates of transcription of promoter contiguous genes vary very dramatically, sometimes over a 20-fold range (Mueller and Getz, 1986; Wettstein-Edwards et al., 1986).

Only two protein components are required for accurate initiation of transcription from *S.cerevisae* mitochondrial promoters *in vitro*. One protein is the 150 kDa core mtRNA polymerase encoded by the *RPO41* gene and the other is a 40 kDa specificity subunit (sc-mtTFB) encoded by the *MTF1* gene. Disruption of the gene for either of these proteins results in loss of mitochondrial DNA and a petite phenotype, underlining their importance for the organism (Greenleaf et al., 1986; Shadel and Clayton, 1995). These two proteins do not show much affinity for each other in the absence of DNA, as they can be separated by mild procedures quite easily (Schinkel et al., 1987). The sc-mtTFB protein structurally resembles a family of enzymes called RNA methyltransferases (Schubot et al., 2001) and together with mtRNA polymerase the protein forms a heterodimer that recognizes mitochondrial promoters and initiates transcription. Shortly after transcription initiation sc-mtTFB dissociates from mtRNA polymerase (Mangus et al., 1994). In DNase footprinting experiments, the core component (mtRNA polymerase) showed random and weak interaction with promoter DNA, the specificity factor none at all, whereas both components together specifically protected a 30-base pair region surrounding the promoter. Similar conclusions were drawn looking at results from gel mobility-shift experiments: no shift was observed with either mtRNA polymerase or sc-mtTFB alone (Mangus et al., 1994; Schinkel et al., 1988). The mtRNA polymerase/mtTFB heterodimer has been studied creating mutants of each protein. The complexity of the interaction surface requires both the entire mtRNA polymerase and mtTFB for functional transcription; in fact none of the protein truncations created retained the ability to interact. On the other hand, point mutations in mtTFB identified at least three regions important for mtRNA polymerase interactions (Cliften et al., 1997). An interaction-defective point mutant in mtTFB was used in a two- hybrid screening to select for suppressing mutations in the core RNA polymerase. Two of the three suppressors were positioned in regions of homology to T7 RNA polymerase. In particular, one of them belongs to the specificity loop, an insertion domain thought to be a major determinant in promoter recognition. This loop is conserved in mtRNA polymerase and it might play a double role: making important

contacts with the DNA promoter and creating protein-protein interactions with the specificity factor (Cliften et al., 2000; Matsunaga and Jaehning, 2004b). Recent work has shown that mtRNA polymerase alone is capable of promoter recognition and transcription initiation in the absence of mtTFB on fully or partially melted open templates *in vitro*. Therefore, mtTFB might be critical for open promoter formation, but not for promoter sequence recognition (Matsunaga and Jaehning, 2004a). This is not particularly surprising due to the homology between mtRNA polymerase and the T7 and T3 RNA polymerases that recognize promoters without additional factors.

The ABF2 protein in yeast mitochondria

The ABF2 protein was initially identified in crude yeast extracts because of its unusual interactions with a yeast origin of nuclear DNA replication, ARS1 (Diffley and Stillman, 1988). ABF2 (also denoted mtTFA) is localized to mitochondria as determined by both subcellular fractionation and *in vivo* immunofluorescence. Yeast abf2$^-$ strains, which lack mtTFA, rapidly lose mtDNA when grown on glucose; on glycerol, where mitochondrial function is essential, the abf2$^-$ mutant is unable to grow at all. The observed phenotype is consistent with a role for sc-mtTFA in mtDNA maintenance (Diffley and Stillman, 1991). The sc-mtTFA amino acid sequence is closely related to the vertebrate, non-histone chromosomal high-mobility group protein HMG1. This HMG protein consists of three domains: an amino-terminal domain, a tandem HMG box domain, an acidic carboxyl-terminal domain (Reeck et al., 1982). mtTFA is composed of the amino-terminal part and the tandem HMG box, but does not contain the carboxyl terminal domain. HMG proteins are capable of binding, wrapping, bending, and unwinding DNA without sequence specificity. *In vitro* studies showed that mtTFA introduces negative supercoils into DNA and it also bends DNA (Fisher et al., 1992). These DNA binding characteristics and the high abundance of this protein suggest that mtTFA may play a role in ordering and compacting mtDNA in much the same way as histones do in the eukaryotic nucleus and the HU protein does in bacteria. HU and mtTFA share no apparent sequence similarity, but mtDNA depletion is rescued by HU. This suggests that mtTFA and HU have similar functions and that mtTFA maintains mtDNA as an architectural factor (Megraw and Chae, 1993). Atomic force microscopic studies have strengthened the idea of mtTFA as the major packaging factor for mitochondrial DNA. mtTFA molecules were shown to be sufficient to compact linear dsDNA and induce a striking collapse of the DNA molecule into a dense

globular complex (Brewer et al., 2003; Friddle et al., 2004). However, mtTFA does not substitute for mtTFB *in vitro*, demonstrating that it is not an alternative specificity factor (Xu and Clayton, 1992).

Transcription initiation in mammals

I*n vitro* studies showed that a partially purified mitochondrial RNA Polymerase (POLRMT) together with pure recombinant mitochondrial transcription factor A (TFAM) was sufficient for initiation of transcription from mitochondrial promoters (Fisher and Clayton, 1985; Fisher and Clayton, 1988). Attempts to use pure POLRMT instead of an *in vivo* purified POLRMT fraction in these assays gave no transcription initiation, suggesting that an additional factor was present together with POLRMT. The specificity factor sc-mtTFB in yeast suggested the possibility of the existence of a homologous protein in humans. Recently, two homologs of sc-mtTFB were identified: mitochondrial transcription factor B1 (TFB1M) and mitochondrial transcription factor B2 (TFB2M) (Falkenberg et al., 2002; McCulloch et al., 2002). The basal human mitochondrial transcription machinery was then reconstituted *in vitro* by combining pure recombinant POLRMT, TFAM and either TFB1M or TFB2M, together with a DNA template containing the mitochondrial promoter sequences HSP and LSP (Falkenberg et al., 2002). TFAM binding induces a structural change of the promoter that is required for POLRMT dependent promoter recognition. A sequence element localized from position -1 to -2 relative to the transcription start site in LSP functionally interacts with POLRMT. Thus, mitochondrial RNA Polymerase contributes specifically to promoter recognition similar to the homologous T7 and T3 RNA polymerases (Gaspari et al., 2004).

Mitochondrial transcription factor A (TFAM)

In addition to mitochondrial RNA polymerase, TFB1M or TFB2M, the 25kDa mitochondrial transcription factor A (TFAM) is absolutely required for transcription initiation from the two strand promoters, the light strand promoter (LSP) and the heavy strand promoter (HSP) (Fisher and Clayton, 1985). In fact, it specifically binds to high affinity sequences upstream of each transcription initiation site and the binding is required for transcriptional activation of the mitochondrial promoters (Fisher et al., 1989; Topper and Clayton, 1989). TFAM is a very abundant protein in mitochondria; it is homologous to sc-mtTFA and is thus a member of the high mobility group (HMG)

proteins. It contains two HMG-box domains, which allow binding, wrapping and bending of DNA without sequence specificity and a 25 amino acid residue carboxyl-terminal tail, which is necessary for transcriptional activation. Therefore, TFAM, but not yeast mtTFA lacking the carboxy terminal tail, serves as a potent activator of transcription. In fact, addition of the human C-terminal tail to the C terminus of sc-mtTFA conferred transcriptional activation function to yeast mtTFA in *in vitro* studies (Dairaghi et al., 1995). Furthermore, the ability of TFAM to rescue mtTFA-deficient yeast strains suggests that these two proteins share the common role of maintaining mtDNA. In fact, TFAM is imported efficiently into yeast mitochondria, processed correctly and rescues the loss of mitochondrial DNA. TFAM has a dual function in human mitochondria, both in transcriptional activation and DNA maintenance (Ekstrand et al., 2004; Parisi et al., 1993). Consistent with this notion, *in vivo* disruption of the mouse *Tfam* gene causes embryonic lethality between E 8.5 and 10.5. These homozygous *Tfam* knockout embryos exhibit severe mtDNA depletion with abolished oxidative phosphorylation; in heterozygous knockouts mtDNA levels are decreased. TFAM is thus a key regulator of mtDNA copy number *in vivo* and is essential for mitochondrial biogenesis and embryonic development (Larsson et al., 1998). A RNA interference (RNAi) study was used to deplete *Drosophila* Kc167 cells of d-TFAM protein. A consequence of the depletion was a marked reduction of mtDNA levels; as in yeast, TFAM is implicated in stabilization of mtDNA by wrapping and condensing DNA. On the other hand, mtRNAs remained unchanged in the *d*-TFAM depleted cells, suggesting that *d*-TFAM is dispensable for transcription in *Drosophila* (Goto et al., 2001). Mitochondrial DNA is complexed with TFAM (Alam et al., 2003); overexpression of the mammalian protein in cell lines or in transgenic mice show that mtDNA levels are nicely correlated with the amount of TFAM, but not with mtDNA transcript levels. Thus, mtDNA copy number regulation can be dissociated from mtDNA expression *in vivo* (Ekstrand et al., 2004; Kanki et al., 2004).

TFB1M and TFB2M

Bioinformatics analyses were used to identify human transcription factors B1 and B2, as homologs of sc-mtTFB (Falkenberg et al., 2002; McCulloch et al., 2002); Tfb1m and Tfb2m are the mouse homologs (Rantanen et al., 2003). These proteins present sequence similarity to a family of rRNA methyltransferases, which dimethylate two adjacent adenosine bases near the 3' end of the small subunit of rRNA. In particular,

TFB1M is closely related to the *Escherichia coli* RNA adenine dymethyltransferase Ksg A (Schubot et al., 2001). This methylation is not essential in bacteria; in fact, lack of methylation confers resistance to the antibiotic kasugamycin. Recent studies have shown that expressing TFB1M from a plasmid in a ksgA$^-$ strain completely reversed the antibiotic resistance phenotype. Furthermore, primer-extension analysis on purified 16S rRNA from *E.coli* ksgA$^-$ expressing TFB1M, showed blockage of extension, indicating that methylation was restored. Thus, TFB1M functions as a mitochondrial transcription factor and a rRNA modification enzyme (Seidel-Rogol et al., 2003). Intriguingly, the yeast mitochondrial small subunit rRNA is one of the few exceptions known in which dimethylation of the two adjacent A residues does not occur (Klootwijk et al., 1975), however, information regarding small subunit rRNA modifications in other mitochondrial systems is sparse. Sequence similarities between sc-mtTFB and rRNA methyltransferases are poor but the crystal structure of sc-mtTFB revealed high similarity to the *E. coli* rRNA methyltransferase ErmC' protein folds. It is therefore possible that a rRNA dimethyltransferase was recruited to the mitochondrial transcription machinery early in evolution, but that the original methyltransferase activity has been lost in *S. cerevisiae*. The relative importance of human TFB1M and TFB2M in mitochondrial transcription and rRNA methylation remains to be established. In the human *in vitro* transcription system, TFB2M is at least two orders of magnitude more active than TFB1M in promoting transcription initiation. It is therefore possible that TFB2M has evolved into the specialized transcription factor, while TFB1M remains the dimethylating enzyme. Some recent RNA interference (RNAi) experiments in *Drosophila* seem to support this idea. RNAi knock down of *d*-TFB2M reduces the abundance of specific mitochondrial RNA transcripts 2 to 8 fold and *d*-TFB1M cannot complement the deficiency in *d*-TFB2M, pointing to specialized roles for the two factors *in vivo*. In striking contrast, neither RNAi knockdown nor overexpression of *d*-TFB1M affects the levels of mitochondrial transcripts, suggesting that *d*-TFB1M does not have a critical role in transcription, but it is rather a rRNA adenine methyltransferase (Matsushima et al., 2005; Matsushima et al., 2004).

Bidirectionality

Bidirectionality is a recurring motif in mitochondrial transcription systems. Bidirectional transcription initiation has been described for chicken (L'Abbe et al., 1991), *Xenopus laevis* (Bogenhagen and Yoza, 1986), mouse (Chang and Clayton, 1986) and human mitochondrial promoters (Chang and Clayton, 1984). In chicken and *Xenopus*, a conserved nucleotide sequence (ACPuTTAT) surrounds each transcription initiation site. In the frog, two bidirectional promoters are situated in the D-loop; each promoter consists of two octamer sequences located on opposite DNA strands and overlapping. In chicken, only one single bidirectional promoter is present. In both cases, the octamers are reminiscent of the nonanucleotide promoter elements of yeast mitochondria. The bidirectional promoter configurations in these species may represent evolutionary intermediate between single unidirectional promoters in yeast and two primarily unidirectional promoters in humans (Shadel and Clayton, 1993) (**Figure 7)**.

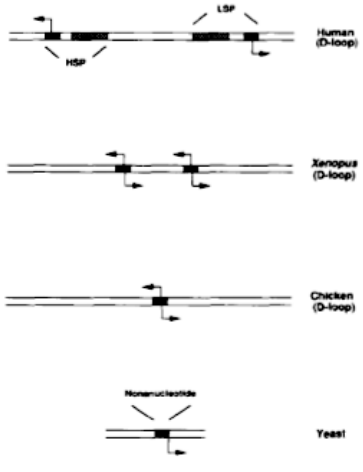

Figure 7. Comparison of mitochondrial promoter elements from yeast (S. cerevisiae) and three vertebrates.

The promoter elements from human, *X. laevis* and chicken mtDNA are shown along with the simple yeast promoter. The D-loop region promoters are the only major promoters in vertebrate mtDNA, while multiple copies of the yeast promoter are found in yeast mtDNA. Human mtDNA contains two divergently oriented major promoters, LSP and HSP. Unlike the primarily unidirectional promoter elements of human and yeast, the promoters of *Xenopus* and chicken are bidirectional. The yeast promoter is simple non anucleotide sequence and has no known bidirectional character (Shadel and Clayton, 1993).

Transcription termination in mammals

In vivo and *in organello* transcription studies have shown that the 20 to 50 fold higher expression of the rRNA genes compared with the downstream mRNA genes transcribed from the heavy strand, involves the differential activity of two independent controlled overlapping transcription units, HSP_1 and HSP_2 (Montoya et al., 1983). Besides being regulated at the level of transcription initiation, this differential expression of the two transcription units involves termination at the border between the 16S rRNA and the $tRNA^{Leu}$ genes (Christianson and Clayton, 1988; Kruse et al., 1989). Binding of mitochondrial transcription termination factor (mTERF) to a tridecamer sequence within the $tRNA^{Leu}$ gene is the key event for this termination. Understanding the functional role of mTERF has acquired special significance after the demonstration that an A→G transition (nucleotide position 3243) in the middle of the mTERF binding site is associated with the MELAS encephalomyopathy and that this mutation dramatically reduces the binding affinity of mTERF for its target sequence *in vitro* (Hess et al., 1991). Recent work has reconstituted mTERF termination activity *in vitro*. The protein efficiently promotes sequence-specific termination in a completely recombinant and highly purified *in vitro* system for mitochondrial transcription. mTERF shows also distinct polarity: complete termination was observed when the mTERF-binding site was oriented in a reverse position relative to the heavy strand promoter, but only partial termination was seen when the binding site was in the forward position. Thus, mTERF is fully active and does not require additional factors to terminate mitochondrial transcription *in vitro* (Asin-Cayuela et al., 2005). However, the role of mTERF *in vivo* still needs to be established; the 3243 mutation has in fact little effect on the relative levels of upstream and downstream mature transcripts from the termination site (Chomyn et al., 1992). Similarity searches and phylogenetic analysis showed that mTERF belongs to a large and complex protein family, called the MTERF family, shared amongst metazoans and plants. Three novel MTERF genes were identified in vertebrates, which all encode proteins with predicted mitochondrial localization: MTERF2, MTERF3 and MTERF4. mTERF (or MTERF1) and MTERF2 are present only in vertebrates, while MTERF3 and MTERF4 are found both in worms and insects and might represent the ancestral genes in metazoans (Linder et al., 2005).
A recent report showed the cloning and characterization of *Drosophila* MTERF3 (D-MTERF3). A D-MTERF knock down phenotype was produced in *D.Mel-2* cultured cells by the RNAi procedure and it did not affect either mitochondrial replication or

transcription. On the contrary, it decreased the translation rate of half of the mitochondrial mRNAs. The decline in translation ranged from about 20% to about 45% for ND1, the most affected polypeptide, suggesting a role for the protein in the mitochondrial translation process (Roberti et al., 2006). The existence of MTERFs suggest that transcription termination is a very well regulated process in mitochondria and further studies, both *in vivo* and *in vitro*, are necessary to shed light on the functions of these proteins.

PRESENT INVESTIGATIONS

The main objective of this thesis was to study the molecular mechanism of mammalian mitochondrial DNA transcription. In particular, the specific aim of each paper was:

PAPER I: to identify and purify novel components, besides TFAM and POLRMT, required for initiation of human mitochondrial transcription; to reconstitute an *in vitro* system for human mitochondrial DNA transcription with recombinant purified proteins.

PAPER II: to characterize the mouse genes for mitochondrial transcription factors B1 and B2 and study their evolution.

PAPER III: to study how mitochondrial promoters are recognized in mammals, utilizing the reconstituted recombinant human and mouse transcription machineries *in vitro*.

PAPER IV: to understand the architectural role of TFAM in mitochondrial DNA maintenance, using stable and inducible human cell lines overexpressing TFAM.

PAPER V: to use the human recombinant mitochondrial transcription system and demonstrate that conserved sequence box II is a sequence-dependent transcription termination element *in vitro*.

PAPER I

Mitochondrial transcription factors B1 and B2 activate transcription of human mtDNA.

Earlier reports have shown that a partially purified mitochondrial RNA polymerase (POLRMT), together with pure mitochondrial transcription factor A (TFAM) could initiate transcription from LSP and HSP *in vitro* (Fisher and Clayton, 1985; Fisher and Clayton, 1988). Attempts to use pure recombinant POLRMT and TFAM in these assays were unsuccessful, suggesting that an additional factor was needed for initiation of transcription. We knew that in yeast only two proteins, the yeast homolog of POLRMT (mtRNA polymerase) and its specificity factor (mtTFB) are required for initiation of transcription. Furthermore, an activity that co-purified with a protein of 40 kDa (mtTFB) and stimulated transcription by interacting with POLRMT had been described in *Xenopus laevis* (Antoshechkin and Bogenhagen, 1995; Bogenhagen, 1996). We reasoned that a homolog of mtTFB was probably necessary for human mitochondrial transcription initiation and started to search the sequence database of the National Center for Biotechnology. We used the putative mtTFB homolog from *Schizosaccharomyces pombe* and identified a predicted human protein that we termed TFB1M. A second human protein, homologous to TFB1M, was found and termed TFB2M. TFB1M and TFB2M showed strong sequence similarity to bacterial rRNA dimethyltransferases. Homologs of both proteins, termed Tfb1m and Tfb2m, were found in the mouse. Mitochondrial localization of these new proteins was shown through confocal microscopy in live cells by expressing fusion proteins between TFB1M/TFB2M and green fluorescent protein (GFP). Both Tfb1m-GFP and TFB2m-GFP had a localization pattern indistinguishable from that of ornithine transcarbamylase (OTC-GFP), a protein that is known to localize in mitochondria. Expression studies were performed on different human tissues and the pattern of expression was similar to the *Tfam* gene, with high levels of expression in heart, liver and skeletal muscle. We developed a pure *in vitro* transcription system in which mitochondrial DNA fragments containing human HSP and LSP were added to recombinant POLRMT, TFAM, TFB1M or TFB2M. We found that the basal mitochondrial transcription machinery consists of three factors: POLRMT and TFAM, together with TFB1M or TFB2M. Level of transcription initiation with TFB1M is at least an order of magnitude lower than with TFB2M. There are also differences in the activation of the two promoters by TFAM, with LSP being active over a broader range

of TFAM concentrations compared with HSP. Several lines of evidence suggest that the TFBMs form heterodimers with POLRMT. In fact, maximal transcriptional activity is seen when TFB2M is present together with POLRMT in a 1:1 molar ratio. Furthermore, purification of POLRMT was only possible when it was coexpressed with either TFB1M or TFB2M. To strengthen this result, we added recombinant TFB2M to TFB2M-immunodepleted mitochondrial extracts and saw that this was not enough to reconstitute transcription. To regain transcription activity, it was necessary to add the heterodimeric POLRMT/TFB2M complex. This result thus suggested that removal of TFB2M resulted in loss of the associated POLRMT.

PAPER II

Characterization of the mouse genes for mitochondrial transcription factors B1 and B2.

After identifying TFB1M and TFB2M we characterized the mouse homologs, Tfb1m and Tfb2m. We mapped *Tfb1m* and *Tfb2m* to mouse chromosome 17 and chromosome 1; the *Tfb1m* gene spans 38.5 kb and consists of seven exons, *Tfb2m* spans 18.2 kb and is divided into eight exons. Another locus for *Tfb2m* was found on chromosome 12, this gene locus contains no introns and lacks the coding sequence corresponding to the first 49 amino acids of the open reading frame (ORF) encoded by the Tfb2m cDNA. The absence of introns and the lack of a methionine codon at the beginning of the truncated ORF suggest that the locus on chromosome 12 is a processed pseudogene lacking the potential to encode a functional Tfb2m protein. Similarly, a human processed pseudogene for *TFB2M* exists on human chromosome 6. Next, we analyzed the promoter regions of both human and mouse genes and saw that they contained several binding sites for the transcription factors NRF-2 and SP1. These factors are already known to regulate *Tfam* gene expression (Virbasius and Scarpulla, 1994) and other nuclear genes, whose products contribute to mitochondrial respiration. Expression studies showed that *Tfb1m* and *Tfb2m* are ubiquitously expressed in a similar way to the human genes. BLAST searches were performed to find TFB1M and TFB2M homologs in other organisms. We found homologs to both proteins in *Drosophila melanogaster,* whereas only was single homolog was found in yeast and *Caenorhabditis elegans*. These findings suggest a duplication event of the *Tfbm* gene in early evolution, indicating that the two factors may have evolved to assume different functions: TFB2M as a transcription factor and TFB1M as a methyltransferase.

PAPER III

The mitochondrial RNA polymerase contributes critically to promoter specificity in mammalian cells.

Mitochondrial extracts from mouse cells can initiate transcription from mouse promoters, but not from human promoters. In a similar way, human mitochondrial extracts can only support transcription from human promoters (Fisher et al., 1989). We decided to employ the observed specificity to address the issue of promoter recognition and the mechanisms of transcriptional initiation in mammalian mitochondria. We speculated that if we had reconstituted transcription from mouse, we could swap individual transcription factors with their human counterparts. The identification of the factor responsible for promoter recognition would be an important step towards a molecular understanding of transcriptional initiation in mitochondria. We cloned cDNAs encoding transcription factors for mouse POLRMT, TFAM and TFB2M. We used the cDNAs to make recombinant baculoviruses and expressed the proteins in insect cells as in the human system. We first investigated the activity of the mouse transcription system on the mouse LSP promoter. Our experiments showed that the mouse system was highly active on the mouse LSP promoter and dependent on the simultaneous presence of mTFAM, mPOLRMT and mTFB2M, just as previously described for the human system. We next verified species specificity in our highly purified recombinant *in vitro* systems. As predicted from the previous reports based on transcription in mitochondrial extracts, the human transcription factors were unable to initiate transcription from a mouse LSP and vice versa. This demonstrated that that the specificity was due to differences in the basal transcription machinery components and not caused by additional factors present in the mitochondrial extracts. We also saw that species specificity was governed by DNA sequences upstream of the transcription start site; transcription initiation was dependent on the interaction of TFAM and POLRMT with two sequence elements: the specific distal promoter element (DPE) and the proximal promoter element (PPE). The distal promoter element (DPE) corresponds to the previously characterized TFAM binding site, while the PPE corresponds to nucleotides –1 and –2 from the transcription initiation site. TFAM binding to DPE introduces specific structural alterations in mtDNA, such as promoter unwinding, which can facilitate promoter recognition by the TFB2M/POLRMT heterodimer at the PPE. On human LSP, we found that mTFAM was at least as active as hTFAM in the stimulation of the human transcription machinery. Furthermore, mTFB2M could

replace hTFB2M, without affecting species-specific promoter recognition. In contrast, mPOLRMT failed to replace hPOLRMT and initiate transcription either in the presence of mTFB2M or hTFB2M. Our analysis therefore demonstrated that it was mPOLRMT and not mTFAM or mTFB2M, which was responsible for the inability of the mouse transcription machinery to initiate transcription at hLSP. A hybrid promoter construct with mTFAM DPE and a single base pair change at position –1 allowed the complete mouse system to initiate transcription from the human LSP, indicating that POLRMT interacts sequence specifically with this region of the mitochondrial promoter. Dnase I footprinting experiments confirmed that the hPORMT/hTFB2M complex requires hTFAM binding in order to recognize and bind human LSP. The POLRMT/TFB2M heterodimer is unable to interact with promoter elements and initiate even abortive transcription in the absence of TFAM. Transcription from human LSP generated two major products: the full length transcript and a short abortive transcript of 4 nucleotides. The production of both products was absolutely dependent on the simultaneous presence of all three proteins. Thus, TFAM has just not evolved to stimulate transcription, but has become an essential part of the transcription machinery. Our finding that POLRMT governs sequence-specific interactions at the PPE suggests that sequence-specific DNA binding may be a general property of mitochondrial RNA polymerases. Mitochondrial RNA polymerase belongs to the family of T7-like RNA polymerases. Promoter recognition by T7RNAP is achieved by the insertion of a specificity loop into the DNA major groove. This specificity loop is present also in mitochondrial RNA polymerase and it may act in the same way at mitochondrial promoters.

PAPER IV

Architectural role of mitochondrial transcription factor A in maintenance of human mitochondrial DNA

The maintenance of mtDNA integrity is essential for normal function of the respiratory chain, which, in turn, is responsible of ATP production. It is now generally accepted that TFAM is absolutely required for transcription initiation, but it also has a role in mitochondrial DNA maintenance. TFAM is an HMG-box protein and thus binds DNA regardless of sequence specificity; it is very abundant and the amount is sufficient to completely cover the mtDNA. Disruption of sc-mtTFA, the TFAM homolog in *Saccharomyces cerevisiae*, leads to loss of mtDNA and respiratory competence. This

phenotype can be rescued by expression of human TFAM, implying a functional homology between human TFAM and sc-mtTFA (Parisi et al., 1993). Furthermore, knock out of *Tfam* gene in mouse and chicken cells causes mtDNA depletion and loss of oxidative phosphorylation. The expression of TFAM was reduced by about 50% and the amount of mtDNA also decreased about half (Larsson et al., 1998; Matsushima et al., 2003). Since mtDNA replication is coupled to transcription, one could argue that TFAM dependent mtDNA maintenance may be related to the formation of RNA primers needed for correct replication initiation at O_H. On the other hand, the unspecific binding capacity of TFAM could play a role in stabilizing and packaging mtDNA, in the same way as other HMG proteins do. To clarify the role of TFAM, we repressed the expression of endogenous TFAM in HeLa cells by RNA interference (RNAi). The amount of human TFAM was decreased maximally at days 3 and 4 and then gradually increased again. The downregulation did not affect the amounts of other nucleus-encoded mitochondrial proteins, confirming that the RNAi interference was specific. Measurements of mtDNA by quantitative PCR showed that the amount of mtDNA fell and rose in parallel with that of human TFAM, indicating that mitochondrial DNA and TFAM are strongly correlated *in vivo*. To see whether an increase in TFAM would affect the amount of mitochondrial DNA, we created two stable and inducible cell lines: the first overexpressing human TFAM deleted of the C tail (TFAM-ΔC-HA), the second overexpressing mouse full length TFAM (TFAM-HA). Both constructs had an HA tag in order to discriminate between the endogenous human TFAM and the overexpressed protein. We saw that overexpression of TFAM increased the amount of mtDNA in parallel. In particular, mtDNA was increased by the overexpression of C tail deleted human TFAM, which does not activate transcription, raising the possibility that the increase in mtDNA does not require the upregulation of transcription. In RNAi experiments, we could replace around 85% of full length human TFAM by TFAM-ΔC-HA, without seeing any reduction in mitochondrial DNA levels. We also showed that TFAM-ΔC-HA has only 1% of the LSP-dependent transcription activity of full-length TFAM in a pure *in vitro* recombinant transcription system. If the majority of TFAM molecules maintained mtDNA through transcription-coupled replication, the amount of mtDNA would decrease. This suggested that the majority of TFAM participates architecturally in mitochondrial DNA maintenance. Furthermore, mitochondria were prepared from overexpressing cell lines, solubilized with NP-40 and separated into insoluble (P1) and soluble (S1) fractions. As previously reported (Alam et al., 2003), both the endogenous and overexpressed TFAMs were mostly recovered from the

insoluble fraction together with mtDNA, supporting the idea of TFAM-mtDNA binding. We therefore concluded that TFAM exists abundantly and that it coats mitochondrial DNA, probably packaging it. Goto *et al.* (Goto et al., 2001) reported that TFAM levels can be substantially reduced by RNAi without significant inhibition of transcription per mtDNA in insect cells; Ekstrand *et al*. (Ekstrand et al., 2004) showed that the role of TFAM in mtDNA maintenance is separate from its function in transcription initiation. In fact, expression of human TFAM in the mouse can directly up-regulate mitochondrial DNA copy number *in vivo*, without increasing mtDNA expression, respiratory chain function or mitochondrial mass.

PAPER V

Conserved sequence box II directs transcription termination and primer formation in mitochondria.

Mammalian mitochondrial DNA (mtDNA) replicates by a mechanism of unidirectional displacement synthesis, initiating from two distinct origins, O_H and O_L (Clayton-model) (Shadel and Clayton, 1997). Synthesis of heavy (H) strand occurs first and gives rise to the so-called displacement loop DNA strand (D-loop). In mouse and human cells, a RNA attached to the newly synthesized H strand has been detected. All these RNAs, upstream of the origin of H strand replication, have common 5' ends at nucleotide 407. This position is identical to the initiation site of LSP, suggesting that all RNAs must be products of transcriptional events. Thus, the light strand promoter (LSP) generates transcripts used in replication priming and for expressing structural genes (Chang and Clayton, 1985; Gillum and Clayton, 1979). The precise alignment of putative primer RNA 3' ends to DNA 5' ends implies that transition from RNA synthesis to DNA synthesis occurs through precise processing at the D-loop. The exact mechanism that leads to the specific transition is not known. However, the RNAs 3' termini are associated with three conserved sequence blocks (CSB I, CSB II, CSB III), which have been recognized in vertebrate mtDNAs (Walberg and Clayton, 1981; Wong et al., 1983). For many years, RNase mitochondrial RNA processing (Rnase MRP) was proposed to be responsible of processing these RNA primers, because of its ability to cleave LSP transcripts *in vitro* at sites, which match the priming sites of leading mtDNA synthesis observed *in vivo* (Lee and Clayton, 1997). In this work, we use a purified recombinant human mitochondrial transcription system and demonstrate that conserved sequence box II (CSB II) is a sequence-dependent transcription termination element *in vitro*. Transcription from LSP generated not only the expected run off

transcript (RO), but also a shorter prematurely terminated transcript (PT). Pulse-chase experiments showed that PT was formed by true termination and not by POLRMT pausing. The PT products terminated ~100 bp downstream of LSP in a region encompassing the conserved sequence element blocks CSB I, CSB II and CSB III. To examine whether these elements could influence PT formation, we created mutations in each CSB box and monitored effects on transcription termination *in vitro*. Mutations of CSB II and CSB III decreased premature transcription termination; in particular CSB II mutation almost abolished PT formation. We thus concluded that PT formation is critically dependent on the CSB II element. To exactly define the 3'-ends of the PT transcripts, we separated transcription reactions on an 8% denaturing urea polyacrylamide gel. Premature transcription termination took place at nucleotide positions 300-282, immediately downstream of CSB II, which spans positions 315-299. To better define the sequence requirements for transcription termination a new series of mutant constructs for CSB II were created. It appeared that sequences within the 3'-end of the CSB II element are required for PT formation, whereas sequences immediately downstream of CSB II do not affect this process. We also saw that CSB II dependent termination was not specific for POLRMT. T7 RNA polymerase was in fact able to produce a terminated transcript of the expected size in a similar way as POLRMT. We concluded that the primary DNA sequence and probably also structural features of CSB II might stimulate transcription termination by both POLRMT and T7 RNA polymerase. Finally, we were able to demonstrate that RNA to DNA transitions took place within a region immediately downstream of CSB II, with a maximum at positions 301-299 and 292-289 of the human mtDNA. These transitions coincide perfectly with the 300-282 region in which we saw premature termination of transcription.

CONCLUDING REMARKS

Our understanding of the molecular mechanism of human mitochondrial transcription has been hampered by the lack of a pure *in vitro* DNA transcription system. The presence, in yeast, of the specificity factor mtTFB prompted us to search for a human homolog, which together with TFAM and POLRMT could activate mitochondrial promoters. Two mammalian homologs TFB1M and TFB2M were identified and we set up a pure *in vitro* transcription system that comprised mtDNA fragments containing HSP and LSP and recombinant TFAM, TFB1M or TFB2M and POLRMT proteins purified after expression with baculovirus in insect cells. With this new system, we started to study the individual components and their role in transcription initiation. In particular, the presence of two factors with strong sequence homology, but maybe different function is intriguing. TFB1M and TFB2M are similar to bacterial rRNA dimethyltransferases; they are both transcription factors, but TFB2M has a 10 fold higher activity *in vitro*. Furthermore, we were not able to detect methyltransferase activity *in vitro* when using different RNA substrates. Therefore, the distinct functions of the two proteins are still unknown. It is unclear if and where these proteins interact with mtDNA. There is one report in which binding of TFB1M to human LSP is shown, but we have not been able to reproduce these findings in our laboratory. In our hands, neither TFB1M nor TFB2M bind to double or single stranded DNA at the promoter regions. Instead, TFB2M is a RNA binding protein (Yonghong Shi, unpublished observation). The protein may therefore interact with newly synthesized RNA and prevent the formation of a RNA/DNA hybrid at the promoter, which could inhibit further rounds of transcription initiation. Looking at their evolutionary history, it is also possible that TFBM2 has lost its ability to bind and methylate RNA during time; instead it has become a more efficient transcription factor than TFB1M. Could then TFB1M complement for TFB2M and vice versa? And if so, which function? Knockouts of both proteins are on going in our laboratory; they will for sure shed some light in understanding the function of these transcription factors *in vivo*. Once we had reconstituted the human mitochondrial machinery, we decided to establish an *in vitro* transcription system with the mouse proteins. We then used a factor-swapping approach to study which components were involved in mitochondrial promoter recognition. Because of the homology of POLRMT with T7 RNA polymerase, we reasoned that POLRMT could be a good candidate. Promoter recognition by T7 RNA

polymerase is achieved by the insertion of a “specificity loop” (amino acids 739-770) into the DNA major groove (-8 to –12bp) and of a flexible “surface loop” (amino acids 93-110) into the minor groove of an A+T rich sequence (-13 to –17bp) (Cheetham et al., 1999). We could identify the specificity loop also in POLRMT and reasoned that sequence-specific DNA binding may be a general property of mitochondrial RNA polymerases. Our experiments demonstrated that POLRMT governs promoter recognition at a proximal promoter element (PPE), position -1 to –2 from the transcription initiation site of LSP. Together with POLRMT, TFAM is essential for transcription initiation, since it binds to the promoter and might cause its unwinding. In future work it would be interesting to investigate which amino acids in the specificity loop of POLRMT are determinants of promoter specificity. In T7 RNA polymerase, a single amino acid at position 748 is the primary determinant of specificity for the bp –11 in the major groove. Substitution of this residue (Asn) with the corresponding residue found in T3 RNA polymerase (Asp) results in a switch in promoter specificity (Raskin et al., 1992). Similar studies between the mouse and human systems could be pursued. The abundance of TFAM together with its unspecific DNA binding activity has suggested that mitochondrial DNA is far from naked (Alam et al., 2003). TFAM is essential for transcription initiation and packaging of human mtDNA by TFAM may be critical for maintaining the mitochondrial genome, as is packaging of yeast mtDNA by sc-mtTFA. Furthermore, mitochondrial DNA and TFAM could be unstable in free form and could need to associate with each other. Our experiments demonstrated that the amount of mtDNA is strongly correlated to the level of TFAM in human cell lines. This correlation was measured on a daily change and the amount of mtDNA was related to the TFAM level on a time scale that appeared to be less than one day. So, mtDNA levels may reach corresponding levels of TFAM within hours. We also found out that mouse full length TFAM (TFAM-HA) and human TFAM deleted of the C tail (TFAM-ΔC-HA) were as active as wild type human TFAM in mtDNA maintenance; we saw that the transcription level was not correlated with the amount of TFAM. Thus, TFAM, which is in functional excess, might maintain the mitochondrial genome through unspecific DNA binding as an HMG box protein. Further speculations are even suggesting the idea of a mitochondrial chromosome or mitochromosome, in which TFAM could package mtDNA in a way similar to histones in the nucleus. Our results are strengthened by another study in HeLa cell lines. In this study, they showed that when mtDNA was depleted with ethidium bromide, TFAM was reduced to the same extent as mtDNA. Thus, TFAM and mtDNA may stabilize each other when bound

together (Seidel-Rogol and Shadel, 2002). Antoshechkin *et al.* reported that *in vitro* chemical cross-linking experiments provided evidence of cross-linked dimers, trimers and tetramers of xl-mtTFA. They also established that xl-mtTFA binds to the promoter proximal site predominantly as a tetramer (Antoshechkin et al., 1997). Further studies on these forms of native TFAM could be pursued to understand if these complexes might have a role in the packaging of mtDNA. Maybe a crystal structure of the protein would be needed. The fact that TFAM is coating mtDNA *in vivo* suggests that maybe remodelling complexes are present in mitochondria. The regulation of chromatin structure is of fundamental importance in the nucleus; activation or repression of gene transcription or DNA replication depends on enzymes that can generate the appropriate chromatin environment. Nulceosomes are displaced in processes that involve histone-modification, ATP-dependent nucleosome remodelling complexes, histone chaperones, etc (Workman, 2006). When mtDNA replication takes place, two new mitochondrial DNA strands are synthesized and TFAM must indeed be displaced and replaced under a controlled mechanism in a similar way to the nucleosomes. How this process occurs is of great interest to us, since future studies might include setting up a human mitochondrial coupled transcription-replication system *in vitro*. Of course, we would like this system to resemble as much as possible the *in vivo* situation. A mitochondrial replisome and a mitochondrial transcription system have already been established. Furthermore, we have shown that CSB II is a sequence-dependent transcription termination element *in vitro*. It is tempting to speculate that this element could function as a loading site for the DNA replication machinery, in a similar way to how transcription switches to replication in the T7 system. During the initiation of bacteriophage DNA replication, the T7 DNA polymerase displaces the RNA polymerase at a specific region and uses the newly synthesized transcript as a primer for DNA synthesis. Therefore, no primer processing is required to initiate T7 bacteriophage DNA replication. Indeed, the high homology of POLRMT and mitochondrial DNA polymerase to the bacteriophage T7 enzymes tempts us to speculate that such a mechanism might be present in mitochondria. Further studies are needed to evaluate this idea and the role of RNase-MRP in this process.

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